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Original Article

Cardioprotection of stevioside on stunned rat hearts: A mechano-energetical study

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ABSTRACT

Background: The sweetener and hypoglycemic properties of stevioside (STV) are well known, as the main component of the plant *Stevia rebaudiana*. Given its extensive use in diabetic patients, it was of interest to evaluate its effects on the most frequent cardiovascular disease, the coronary insufficiency.
Purpose: To study whether STV could be cardioprotective against ischemia-reperfusion (I/R) in a model of "stunning" in rat hearts.
Study design: A preclinical study was performed in isolated hearts from rats in the following groups: non-treated rats whose hearts were perfused with STV 0.3 mg/ml and their controls (C) exposed to either moderate stunning (20 min I/45 min R) or severe stunning (30 min I/45 min R), and a group of rats orally treated with STV 25 mg/kg/day in the drink water during 1 week before the experiment of severe stunning in the isolated hearts were done.
Methods: The mechano-calorimetrical performance of isolated beating hearts was recorded during stabilization period with control Krebs perfusion inside a calorimeter, with or without 0.3 mg/ml STV before the respective period of I/R. The left ventricular maximal developed pressure (P) and total heat rate (Ht) were continuously measured.
Results: Both, orally administered and perfused STV improved the post-ischemic contractile recovery (PICR, as % of initial control P) and the total muscle economy (P/Ht) after the severe stunning, but only improved P/Ht in moderate stunning. However, STV increased the diastolic pressure (LVEDP) during I/R in both stunning models. For studying the mechanism of action, ischemic hearts were reperfused with 10 mM caffeine-36 mM Na^+-Krebs to induce a contracture dependent on sarcorreticular Ca^{2+} content, whose relaxation mainly depends on mitochondrial Ca^{2+} uptake. STV at 0.3 mg/ml increased the area-under-curve of the caffeine-dependent contracture (AUC-LVP). Moreover, at room temperature STV increased the mitochondrial Ca^{2+} uptake measured by Rhod-2 fluorescence in rat cardiomyocytes, but prevented the $[Ca^{2+}]$m overload assessed by caffeine-dependent SR release.
Conclusions: Results suggest that STV is cardioprotective against I/R under oral administration or direct perfusion in hearts. The mechanism includes the regulation of the myocardial calcium homeostasis and the energetic during I/R in several sites, mainly reducing mitochondrial Ca^{2+} overload and increasing the sarcorreticular Ca^{2+} store.

Abbreviations: AUC, area under curve; C, control perfusion; F/Fo, relative fluorescence; Ht, heat rate production; I/R, ischemia/reperfusion; LDH, lactate-deshidrogenase; LVEDP, left ventricular end diastolic pressure; LVP, left ventricular pressure; mKATP, mitochondrial ATP-dependent potassium channels; mNCX, mitochondrial sodium/calcium exchanger; NCX, sarcolemmal sodium/calcium exchanger; P, maximal pressure development; PICR, postischemic contractile recovery; P/Ht, total muscle economy; R-caff-36 Na^+, reperfusion with Krebs + caffeine 10 mM and 36 mM Na^+; RyR, ryanodine receptors; SERCA, sarcoplasmic reticulum calcium pump; SR, sarcoplasmic reticulum; STV, stevioside

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Introduction

Stevioside is a non-caloric sweetener extracted from the leaves of *Stevia rebaudiana* (Bertoni) (Asteraceae), a native plant from Paraná in Brazil, Paraguay and the Northeast of Argentina. This hydrophilic diterpenoid glycoside is 300 times sweeter than sugar and concurrently a good reductor of postprandial blood glucose in type-2 diabetic patients (Gregersen et al., 2004). It was demonstrated that when orally administered to pigs stevioside was strongly converted into its aglycon steviol by the colonic bacteria (Geuns et al., 2003). It was found that steviol but not stevioside is the responsible to reduce glucose absorption (Koyama et al., 2003).

Stevioside (STV) was also described as hypotensor and vasodilator (Chan et al., 1998; Tirapelli et al., 2010), as diuretic (Melis, 1995) and bradycardic (Humboldt and Boech, 1997) in preclinical and clinical studies. It reduced the vascular resistance by inhibition of calcium influx (Melis and Sainati, 1991; Lee et al., 2001; Liu et al., 2003). On line with this, we described the intestinal antispasmodic effect of STV associated to a non-competitive blockade of Ca^{2+}-influx to smooth muscle cells (Matera et al., 2012). Both effects, bradycardia and Ca^{2+} channels blockade allow us to hypothesize that STV could reduce the Ca^{2+} overload induced during ischemia/reperfusion (I/R). Depending on the duration of ischemia (I), hearts could suffer either a ``stunning" characterized by reduced contractile recovery and the develop of diastolic contracture during R, or an infarction with necrotic areas and loss of contractile activity. Two previous studies described that isosteviol, a derivative compound, induced cardioprotection. In one of them isosteviol was perfused from 1 to 10 μmol/l in isolated guinea-pig hearts exposed to I/R, and the cardioprotection was partially attributed to the opening of the mitochondrial KATP channels (mKATP) like in myocardial preconditioning (Miura et al., 2001). In the other one, Xu et al. (2007) demonstrated that isosteviol intravenously injected before occluding the left coronary artery reduced the size of infarct and the accumulation of injury marker enzymes such as LDH and creatine-kinase. These effects observed in a drastic model of I/R were also partially inhibited by blocking the mKATP (Xu et al., 2007). Since *Stevia rebaudiana* and the STV are used as sweetener and hypoglycemic agents in diabetic patients, it was of interest to assess whether STV could prevent the consequences of brief episodes of myocardial ischemia. These episodes are frequently the result of vascular occlusions in diabetic or non-diabetic people over 60 years old, and Phytotherapy could be useful to prevent them. So, we studied the effects of STV on the mechano-energetical behaviour of isolated rat hearts exposed to two degrees of stunning. These models provide respectively medium and low contractile recovery and mitochondrial metabolic changes, which drive to alterations in muscle economy and Ca^{2+} handling. These *ex vivo* models resemble the clinical situations of a transient coronary obstruction, after which it is required a prevention to avoid further ischemic complications to myocardium.

Contractile and calorimetric output of the isolated heart provides information about the energetic of Ca^{2+} handling and muscle economy. Global I/R induces contractile and energetic dysfunction, since metabolism and calcium homeostasis are affected during R (Bolli and Marban, 1999; Consolini et al., 2007; Valverde et al., 2010; Garcia-Dorado et al., 2014). Myocardial ischemia reduces the metabolic restoration of ATP and the associated heat fraction, but also it reduces the ATP pool compartmentalized and necessary to extrude Ca^{2+} from cytosol (Guzun et al., 2015), both of which become in diastolic contracture during I (Consolini et al., 2004, 2007). Still more contracture appears during first min of R, when the concentrations of H^+, Na^+ and Ca^{2+} increases in cytosol until restoration of mitochondrial metabolism (Schafer et al., 2001; Valverde et al., 2010). With the reperfusion, the sarcoplasmic reticulum Ca^{2+} pump (SERCA), the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) and the mitochondrial transporters (Ca uniporter, mNCX, mKATP and mPTP) play the main roles to restore the cellular Ca^{2+} homeostasis (Motegi et al., 2007; Murphy and Steenbergen, 2008; Bernardi and Di Lisa, 2015; Mattiazzi et al., 2015). The isolated physiologically perfused whole heart allows to study the simultaneous and continuous mechano-energetical behaviour before and during the no-flow ischemia and reperfusion. Then, the aim of this work was to evaluate the effects of STV on the stunning due to two degrees of I/R, without infarct. STV was administered either *ex vivo* and *in vivo* to see whether the effects are due to STV or to some metabolite. Also, the effects of STV on cellular Ca^{2+} handling were explored in isolated cardiomyocytes.

Methods

Animals

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as was recommended in the Guide for Care and Use of Animals (NIH Nro publication # 85-23 revised in 1985 and 1996, National Academy Press, Washington DC, USA), according to the Resolution 1047 anexo II of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) de la República Argentina, 2005.).

Isolated heart preparation and contractile measurements

Adult Sprague-Dawley rats (200 a 250 g weight) of either sex were heparinized (non-fractioned heparine 2000 IU) and anesthetized with 25% urethane (0.6 ml by 100 g, via i.p.). The animals were spontaneously breathing during the general anesthesia. Hearts were rapidly excised and retrograde perfusion through coronary arteries by the Langendorff method was done, as previously described (Consolini et al., 2007; Ragone and Consolini, 2009). Hearts were perfused with control Krebs (C) at 37 °C and at a constant flow rate of 7 ml min^{-1} g^{-1}, by a peristaltic pump (Gilson Minipuls, France). This perfusion flow was calculated by the equation $CF = 7.43*HW^{0.56}$ (where CF is coronary flow and HW is the heart weight), valid for different species and recommended to prevent heart edema caused by high flow rate of saline perfusion (Dhein, 2005). This flow was sufficient to develop an optimal maximal pressure (P) without a significant edema, as described in other work (Colareda et al., 2016). The experimental conditions (working temperature, heart rate and I/R periods) were similar to those of previous studies (Ragone and Consolini, 2009; Consolini et al., 2011; Bonazzola et al., 2014; Ragone et al., 2015; Colareda et al., 2016) in which cellular mechanisms had been characterized during stunning of rat hearts. Atria were removed and the spontaneous beating was stopped by applying pressure on the focus in the interventricular septum. A latex balloon was introduced in the left ventricle, connected by a flexible cannula to a Bentley DEL900 or a Statham P23db pressure transducer. While continuously perfused, the heart was introduced into the calorimetrical chamber, which was closed and submerged in a bath kept at controlled temperature of 37.0 ± 0.01 °C. Rat hearts were electrically stimulated with 5 V–5 ms at 3 Hz, by means of two electrodes connected to an electrical stimulator (Letica LE12406, Spain or Grass SD9, USA). The isovolumic left intraventricular pressure (LVP) was continuously recorded at optimal volume, as well as the total heat rate. The LVP was recorded during all the experiment either in a PowerLab 2/26 two channels digital acquisition system (AD Instruments, Australia) or in a Grass polygraph of 8 channels (Grass Instruments, Quincy, MA, USA) with A/D acquisition (TL-1 DMA Axon Instruments INC., Foster City, CA, USA). The maximal pressure development (P) of a contraction was calculated from the difference between the peak in LVP recording and the diastolic level (left ventricular end diastolic pressure (LVEDP)). During I and R there were calculated the changes in diastolic pressure over the preischemic condition in Krebs-C (ΔLVEDP), in mmHg, as an estimation of diastolic contracture. Also, during I/R, the pressure development during a contraction (contractility) was expressed as a percentage of the steady

initial value of P in each heart.

Calorimetrical measurements

The calorimeter was previously described (Consolini et al., 2007; Ponce-Hornos et al., 1982; Ponce-Hornos et al., 1995; Bonazzola et al., 2014). The internal chamber has two ceramic modules with 127 thermosensitive units (Melchor Thermoelectrics) each one, which detect changes in temperature between the inside (heart) and the outside (bath). For details of calibration with a constant electrical power on the muscle at the end of the experiment, and calorimetrical base lines, see the previous publications (Consolini et al., 2001; Consolini et al., 2007; Ragone et al., 2015). The calorimeter was submerged in a water bath at controlled temperature in connection with other two baths for heating the perfusates at the same temperature. Heat outputs were recorded simultaneously to the signal of LVP in the same acquisition system. Before and after introducing the heart, the calorimetrical signal was continuously measured in the presence and the absence of perfusion, which then were used as a base-line in order to be substracted from the total heat signal in the presence of the heart. Consequently, the total heat rate (Ht) was calculated from the difference between those signals, being finally expressed in mW g^{-1} of wet weight. Also, the ischemic and post-ischemic measurements were expressed as a percentage of the steady initial Ht. Finally, the muscle economy was calculated as the P/Ht ratio.

Experimental protocols

After about 40 min of stabilization with Krebs-C, an ``initial control value" of P and Ht was recorded, and then hearts were exposed to different treatments followed by a period of no-flow ischemia (I) and 45 min of reperfusion (R) at 37 °C and 3 Hz of heart rate. The period of I was characteristic of each model, as follows: 20 min in the moderate stunning model, and 30 min in the severe stunning model. These models were previously characterized in hearts perfused only with control Krebs-C solution (Ragone et al., 2015; Colareda et al., 2016).

Stevioside (STV) was assessed as cardioprotective in both models of I/R, by perfusing it at a concentration (0.3 mg/ml) which was chosen from a previous work in which plasmatic concentration was measured during hypoglycemic effect (Gregersen et al., 2004). Also this concentration was effective as an intestinal antispasmodic (Matera et al., 2012). In the protocols, STV was perfused during 20 min before inducing the no-flow ischemia and remained during this period in contact with the heart, but then it was removed during reperfusion. As a positive control, in the model of moderate stunning it was perfused 10 μM clonazepam (Clzp), a cardioprotective drug which blocks the mNCX (Cox and Matlib, 1993). In the case of severe stunning it was perfused 30 μM diazoxide (Dzx) a known cardioprotective drug inhibitor of the mKATP channels (Garlid et al., 2003), as a positive control. Also, it was done a protocol to evaluate the ``*in vivo*" administration of STV, by adding 25 mg STV/kg/day in the drink water during one week, in agreement to the use as hypoglycemic (Jeppesen et al., 2003).

In order to understand whether STV had an effect on the sarcorreticular Ca^{2+} content, the ischemic hearts perfused with STV 0.3 mg/ml and those of a control group (C) were exposed to ischemia and afterwards reperfused with Krebs containing 10 mM caffeine and 36 mM Na^+ (R-caff-36 Na^+). This intervention was classically used to release Ca^{2+} from the sarcoplasmic reticulum (SR) proportionally to its content (Bers, 2001), and prevents the Ca^{2+} efflux by the sarcolemmal sodium-calcium exchanger (NCX) due to the reduced Na^+ gradient (Consolini et al., 2007).

Isolation of cardiac myocytes

Ventricular myocytes were isolated from adult rat hearts (200–280 g weight) as previously described (Bridge et al., 1990; Ragone et al., 2015). Once the animal was anesthetized the heart was quickly removed and placed in a Langendorff system. Heart was perfused with a modified Krebs-24-HEPES solution virtually free of Ca^{2+} for 5 min. After that, the solution was changed to Krebs-24-HEPES with 50 μM $CaCl_2$, 0.1 mg/ml collagenase P (Roche) and 0.02 mg/ml protease XIV (Sigma). After 14 min the solution was changed again to an enzyme free-50 μM Ca^{2+} Krebs-24 HEPES solution. All perfusion solutions were bubbled with O_2 and maintained at 37 °C. Following this, the ventricles were removed and minced, and the pieces were shaken in the low-Ca^{2+} solution for ten min and then filtered. The Ca^{2+} concentration was raised in steps to 1 mM Ca^{2+} and myocytes were stored for up to 6 h in this HEPES-buffered saline solution.

Confocal microscopy with cardiomyocytes

In order to measure changes in the cytosolic free Ca^{2+} signals, isolated rat cardiomyocytes were loaded with 12 μM Fluo-4 AM (Molecular Probes/Invitrogen, Carlsbad, CA, USA) during 15 min at 37 °C. To measure changes in the mitochondrial free Ca^{2+} signals other group of cells were loaded with 3 μM Rhod-2 AM (Molecular Probes/Invitrogen, Carlsbad, CA, USA) during 1 h at 4 °C, followed by a wash-up at 37 °C during at least 1 h in order to load the fluorophore only in the mitochondrial compartment (Ragone et al., 2013; Trollinger et al., 1997). After this, myocytes were placed in a laminin-precoated perfusion chamber and superfused with Krebs-24 HEPES solution containing 2 mM Ca^{2+} until stabilization. Changes in fluorescence from resting cells were recorded in a confocal microscope Leica SP5 (Leica Microsystems, Mannheim, Germany). Data were analyzed by using the Leica LAS AF Lite v. 2.2.1 software. When loaded with Fluo-4, cells were excited at 488 nm, and the changes in fluorescence emission were detected at wavelengths higher than 505 nm in a defined area by cell (ROI). When loaded with Rhod-2, cells were excited at 540 nm, and the changes in fluorescence emission from one ROI by cell were monitored at wavelengths higher than 560 nm. Results were expressed as the self-ratio emission fluorescence intensity (F/F_o). Changes in F/F_o were calculated over time by non-linear adjustment of the base-line obtained during C perfusion at the start and the end of each protocol, by using Origin 7.0 (OriginLab Corporation, Northampton, MA, USA). Signals were recorded each 20 s during 25 min while myocytes were perfused with different protocols, beginning with control Krebs-24 HEPES solution (C) during 5 min, followed by C + 0.3 mg/ml stevioside (C + STV) during 5 min, then by C with 10 mM caffeine-36 mM Na (C-caff-low Na) in the presence of STV by 10 min, and finally return to C by 5 min. Another analogue protocol in the absence of STV was done (control). For each protocol two series were evaluated, one with Fluo-4 and another with Rhod-2.

Solutions and drugs

Hearts were perfused with Krebs-C (in mM): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, and 6 dextrose, bubbled with 95% O_2–5% CO_2. Stevioside (STV, 99.8% purity, from Tanki S.A., Argentina) was prepared as acqueous solution at 0.3 mg/ml the day of the experiment. Also, caffeine (ICN, Costa Mesa, CA, USA) was directly dissolved in Krebs the day of the experiment. Krebs solution with 10 mM caffeine and 36 mM Na^+ was kept isosmotic with Krebs-C by adding 217 mM sucrose.

For cardiomyocytes isolation the solution was (in mM): 126 NaCl, 4.4 KCl, 1.0 NaH_2PO_4, 5 $MgCl_2$, 24 HEPES, 22 dextrose, 20 taurine, 5 creatine, 0.5 Na-pyruvate, adjusted with NaOH to pH 7.4 and bubbled with O_2 100%. During the experiment cardiomyocytes were superfused in Krebs-24-HEPES solution with the following composition (in mM): 126 NaCl, 4.4 KCl, 1 $MgCl_2$, 24 HEPES, 2 $CaCl_2$ and 11 dextrose, adjusted with NaOH to pH 7.4 and bubbled with O_2 100%. For Fluo-4 experiments 0.5 mM probenecid was added.

Statistical analysis

Results were expressed as mean ± SEM. Multiple comparisons by two-way ANOVA for repeated measures (factors were treatment and time) were done for the respective groups of experiments. Also, paired *t*-tests and one-way ANOVA were used when appropriate. Bonferroni paired post-tests were done among the treatments when a significant difference was found by ANOVA, and their results are shown in each figure. Always a significance level of $p < 0.05$ was considered. All statistical analyses were performed by using the Graph Pad Prism v.4 software.

Results

Effects of stevioside in rat hearts exposed to moderate stunning

Hearts perfused with control Krebs had an initial maximal pressure development (P) of 77.8 ± 10.0 mmHg and a diastolic pressure (LVEDP) of 10.1 ± 0.9 Hg, and released an steady heat rate (Ht) of 17.9 ± 1.4 mW g^{-1} ($n = 8$). The period of 20 min I–45 min R induced a moderate stunning in non-treated rat hearts (C group) with a post-ischemic contractile recovery (PICR) of about 60% of the initial P (Fig. 1A). The presence of 0.3 mg/ml stevioside (STV) did not significantly modify P, LVEDP or Ht (respectively 69.9 ± 8.7 mmHg, 11.3 ± 2.5 mmHg and 16.7 ± 1.4 mW g^{-1}). Perfusion of STV before I also did not change the PICR obtained during reperfusion with Krebs-C (R) although it slowed contractile recovery. Contrarily, the positive control with 10 μM clonazepam (Clzp) improved PICR to about 100%. Simultaneously, during R control hearts recovered the total heat rate (Ht) when metabolism and active transport mechanisms were re-activated with the consequent energetic consumption. Fig. 1B shows that both, STV and Clzp reduced Ht of rat hearts during R with respect to the control group. When comparing contractility with the energetic output, the P/Ht ratio shows the total muscle economy. Fig. 1C shows that control hearts slightly reduced P/Ht during R regarding the pre-ischemic condition, but STV and Clzp significantly increased it, showing a cardioprotective effect. Nevertheless, Table 1 shows that, unlike hearts non-treated (C) or perfused with Clzp which reduced the diastolic pressure (ΔLVEDP) during I and slightly increased it during R, STV increased the diastolic pressure during all the period of I/R.

Effects of stevioside in rat hearts exposed to severe stunning

The period of 30 min I–45 min R induced a severe stunning in non-treated rat hearts (C group) with a PICR of about 15% of the initial P (Fig. 2A). The presence of 0.3 mg/ml stevioside (STV) before and during I significantly increased the PICR to about 35% of initial P at the end of R, while the positive control with 30 μM diazoxide (Dzx) improved PICR to about 85% of initial P. Simultaneously, STV increased the total muscle economy (P/Ht) from about 14% (C group) to near 60% (STV group) and Dzx to 125% of initial, indicating a better contractile recovery with low energetic consumption (Fig. 2B). This was an important cardioprotective effect of both, STV and Dzx, in spite of the increased diastolic contracture (ΔLVEDP) induced by perfusing 0.3 mg/ml STV during severe I/R while Dzx reduced it (Table 1).

When rats were orally treated with STV (25 mg/kg/day in the water drink during one week) the isolated hearts exposed to severe ischemia (30 min I and 45 in R) recovered P to about 36% of initial, more than the respective group of control hearts and the same than when STV was perfused in hearts (Fig. 2C). Moreover, STV improved the total muscle economy to about 42% of initial (Fig. 2D) but it maintained the diastolic contracture of C-hearts, preventing the increase seen in hearts perfused with STV before I/R (Table 1).

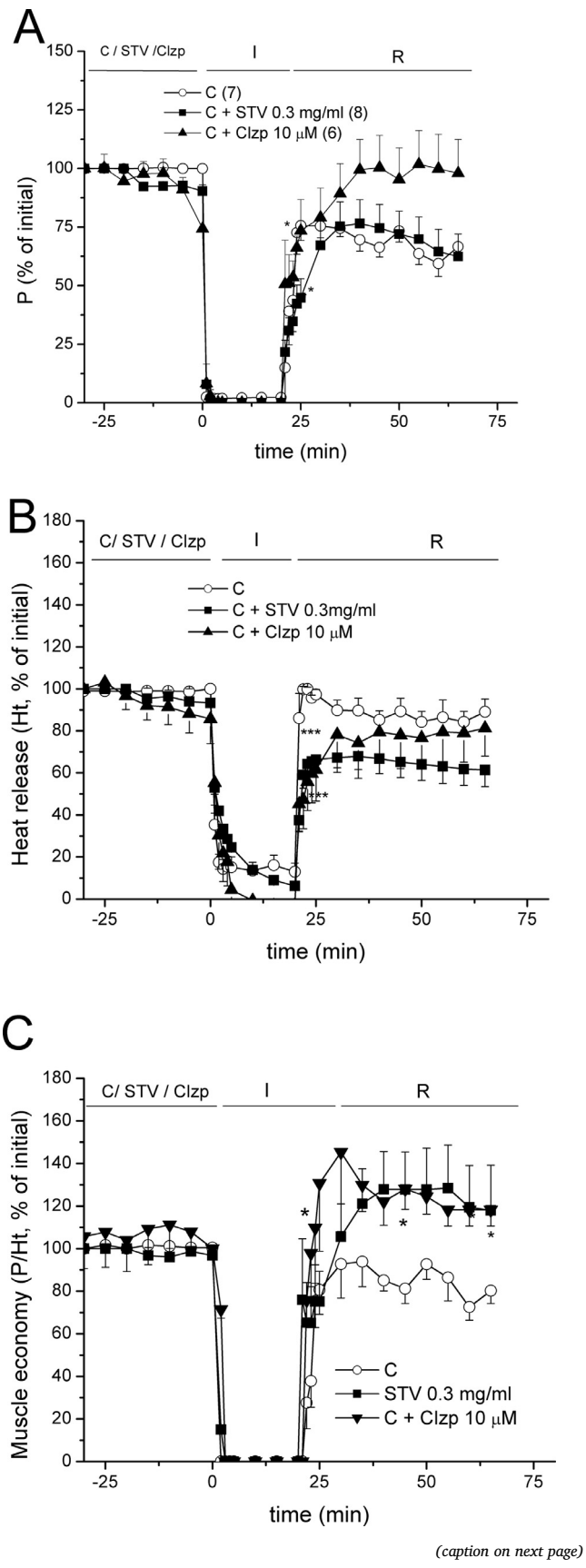

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Fig. 1. Effects of 0.3 mg/ml stevioside (STV) on the maximal pressure development (P) (as % of the initial value, **A**), total heat rate (Ht) (as % of initial value, B) and muscle economy (P/Ht as % of initial value, C) of rat hearts exposed to 20 min ischemia (I) and 45 min reperfusion (R) in a moderate stunning model, in comparison with non-treated hearts (Control). Positive control of hearts perfused with 10 μM clonazepam (Clzp) was also shown. STV and Clzp were perfused before I and remained during it. Results are shown as media ± SEM (n are indicated in panel A) (two-way ANOVA's by treatment: F = 16.86, F = 23.35 and F = 24.73, all $p < 0.0001$ respectively for **A, B** and **C**; by time: F = 86.23, 52.29 and 52.78, respectively for **A, B** and **C**, all $p < 0.0001$. Post-tests $^*p < 0.05$ vs. Control).

Table 1
Changes in the left ventricular end diastolic pressure (ΔLVEDP in mmHg) over the pre-ischemic value induced by ischemia and reperfusion in rat hearts exposed to the models of moderate or severe stunning (see the text). Before ischemia, the hearts were perfused with control Krebs (C) or stevioside 0.3 mg/ml (C + STV) and another group was treated with STV 25 mg/kg/day orally during one week before ($^*p < 0.05$ vs C by *t*-tests or by Bonferroni post-test after ANOVA, respectively for moderate and severe stunning, number of experiments in parenthesis).

Treatment	5 min I	End of I	5 min R	45 min R
Moderate stunning at 37 °C:				
C (7)	–3.5 ± 0.9	–0.5 ± 0.8	7.7 ± 1.9	1.7 ± 0.6
C + STV 0.3 mg/ml (8)	12.2 ± 3.7*	30.6 ± 5.8*	60.2 ± 3.7*	53.5 ± 6.3*
C + Clzp 10 μM (6)	–10.0 ± 4.9	–7.1 ± 4.9	6.0 ± 2.9	4.3 ± 2.7
ANOVA	F = 10.96	F = 20.19	F = 108.4	F = 46.02
	$p = 0.0008$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Severe stunning at 37 °C:				
C (6)	–6.3 ± 3	7.5 ± 3.8	17.9 ± 2.6	16.2 ± 3.8
C + STV 0.3 mg/ml (5)	13.6 ± 7.8*	25.0 ± 8.7	81.8 ± 8.6*	66.4 ± 6.5*
C + STV oral 25 mg/kg (5)	–6.7 ± 4.9	6.2 ± 3.1	21.2 ± 8.3	10.9 ± 0.8
C + Dzx 30 μM (5)	–1.4 ± 1.4	–0.8 ± 2.3	9.4 ± 3.5	4.9 ± 1.5
ANOVA	F = 3.94	F = 4.60	F = 28.69	F = 51.35
	$p = 0.0265$	$p = 0.0156$	$p < 0.0001$	$p < 0.0001$

Effects of stevioside on the sarcorreticular Ca^{2+} content

To evaluate whether the cardioprotective effects of STV were due to an increase in the sarcorreticular (SR) Ca^{2+} content, some experiments were performed in which after the treatment with STV or Krebs-C and ischemia, the hearts were reperfused with Krebs containing 10 mM caffeine, in order to release the SR Ca^{2+}, and 36 mM Na^+ to minimize the efflux through the NCX (Krebs-caff-low [Na^+]). Such protocol of reperfusion was applied to both models: moderate and severe stunning. In all cases, R with Krebs-caff-low [Na^+] induces a disappearance of beating and a contracture evidenced by the maintained increase in pressure over the ischemic level (ΔLVP), a rise in Ht associated to the myofilaments interaction, Ca^{2+} cycling through the SR and mitochondria along with the caffeine activation of RyR2 channels, and the Ca^{2+} removal mechanisms for relaxation (Consolini et al., 2007).

Fig. 3A shows that in the moderate stunning STV reduced the relaxation rate of the contracture induced by Krebs-caff-low [Na^+] (ΔLVP fell more slowly over time) and increased the area under the curve of contracture (AUC-ΔLVP) (Table 2). The simultaneous heat rate (Ht) was not changed by STV (Fig. 3B) as neither the AUC-Ht (Table 2). In the severe stunning, the ΔLVP was increased by STV during I and during the caffeine-low [Na^+] reperfusion, without changing the relaxation rate (Fig. 3C). The AUC-ΔLVP had a non significant tendency to increase in hearts treated with STV (Table 2). The Ht and the AUC-Ht associated to the contracture were not modified by STV in this model (Fig. 3D, Table 2).

The effect of STV on the SR Ca^{2+} content was also evaluated in the well oxygenated isolated cardiomyocytes without electrical stimulation. The change of perfusion from control HEPES-Krebs (C) to HEPES-Krebs with 10 mM caffeine-36 mM Na^+ induced a transient increase in the relative fluorescence (ΔF/Fo) signal of Fluo-4 followed by an abrupt fall to basal level (Fig. 4A), at the time in which the ΔF/Fo of Rhod-2 was slowly increased (Fig. 4B). Control condition shows that caffeine induced the SR Ca^{2+} release to cytosol (Fluo-4 signal), but mitochondria slowly uptake this Ca^{2+} (Rhod-2 signal). Addition of STV did not change the raise and fall of cytosolic Ca^{2+} induced by Krebs-caff-low [Na^+] (Fluo-4 ΔF/Fo) (Fig. 4A). However, STV increased the Rhod-2 ΔF/Fo before applying the Krebs-caff-low [Na^+] perfusion and also sped up the mitochondrial uptake during it, while reduced the final increase in free [Ca^{2+}]m during the Krebs-caff-low [Na^+] perfusion and the turning back to C (Fig. 4B). Results suggest that in non-ischemic cardiomyocytes STV increased the mitochondrial Ca^{2+} cycling but reduced the mitochondrial Ca^{2+} overload.

Discussion

This work describes direct effects of STV on two models of stunning, moderate and severe, in isolated rat hearts. Myocardial contractility and the energetic behaviour were measured during ischemia and reperfusion (I/R). Results show that this frequently consumed sweetener improved the myocardial contractile and energetic recovery after a transient and a more severe ischemia, becoming in a cardioprotective agent. Some aspects of its mechanism were characterized.

It is known that ischemia and reperfusion becomes in several types of myocardial contractile dysfunction, in a range from the stunning to the infarct, and electrical alterations which generate arrhythmias. The experimental models to study these pathologies are based in the isolated perfused heart, since it gives the possibility of simulating the cessation or reduction in perfusion flow (Consolini et al., 2001, 2007). In the last years, the mouse isolated perfused heart was used to simultaneously study the changes in contractility, [Ca^{2+}] in cytosol and sarcoplasmic reticulum (SR), and the action potential during I/R in a stunning model (Valverde et al., 2010). Ischemia induces reduction in the duration of action potential (Cole et al., 1991) causing less Ca^{2+} influx and activation of ryanodine receptors (RyR2) (Fauconnier et al., 2013). Valverde et al. (2010) explained that during I the SR maintains the Ca^{2+} store and during the first min of R the SR quickly loses it to cytosol, becoming in an increased diastolic contracture. The subsequent Ca^{2+} induced-Ca^{2+} release events of SR are reduced during reperfusion as well as the Ca^{2+} transients, thus explaining the reduced contraction (stunning). Also, I/R induces an energetic debt, because of reduction in aerobic metabolism and ATP synthesis. We described that ischemic rat hearts reduced the total muscle economy (P/Ht) during R, due to a lower contraction with diastolic contracture and high energetic cost evidenced by the high heat release (Consolini et al., 2007). We also demonstrated the important role of mitochondria to regulate the SR content and the postischemic contractile recovery (PICR) with Ca^{2+} extrusion through the mitochondrial Na^+/Ca^{2+}-exchanger (mNCX) (Ragone and Consolini, 2009; Consolini et al., 2011; Ragone et al., 2013; Ragone et al., 2015). With this knowledge, we analyzed the present results in isolated rat hearts perfused with STV at a concentration in which it demonstrated antispasmodic effect in isolated intestine associated to Ca^{2+} influx inhibition (Matera et al., 2012). Also, the cardioprotective effect was found in rats treated with STV in drinking water, a condition that imitates the human consumption of this sweetener

The results obtained in the moderate stunning model (Fig. 1) show that STV improved the recovery of total muscle economy (P/Ht), but maintained the PICR and increased the diastolic contracture during I/R (Table 1). Contrarily, the inhibitor of mNCX clonazepam increased PICR and P/Ht and reduced LVEDP, in agreement with the increase in [Ca^{2+}]m and activation of metabolism and ATP synthesis (Cox and Matlib, 1993). These results suggest that STV did not change the SR Ca^{2+} release but reduced the mitochondrial metabolism, and increased the diastolic Ca^{2+} leak to cytosol. To understand the Ca^{2+} homeostasis during R, ischemic rat hearts were reperfused with 10 mM caffeine-36 mM Na-Krebs, since this caffeine concentration induces opening of

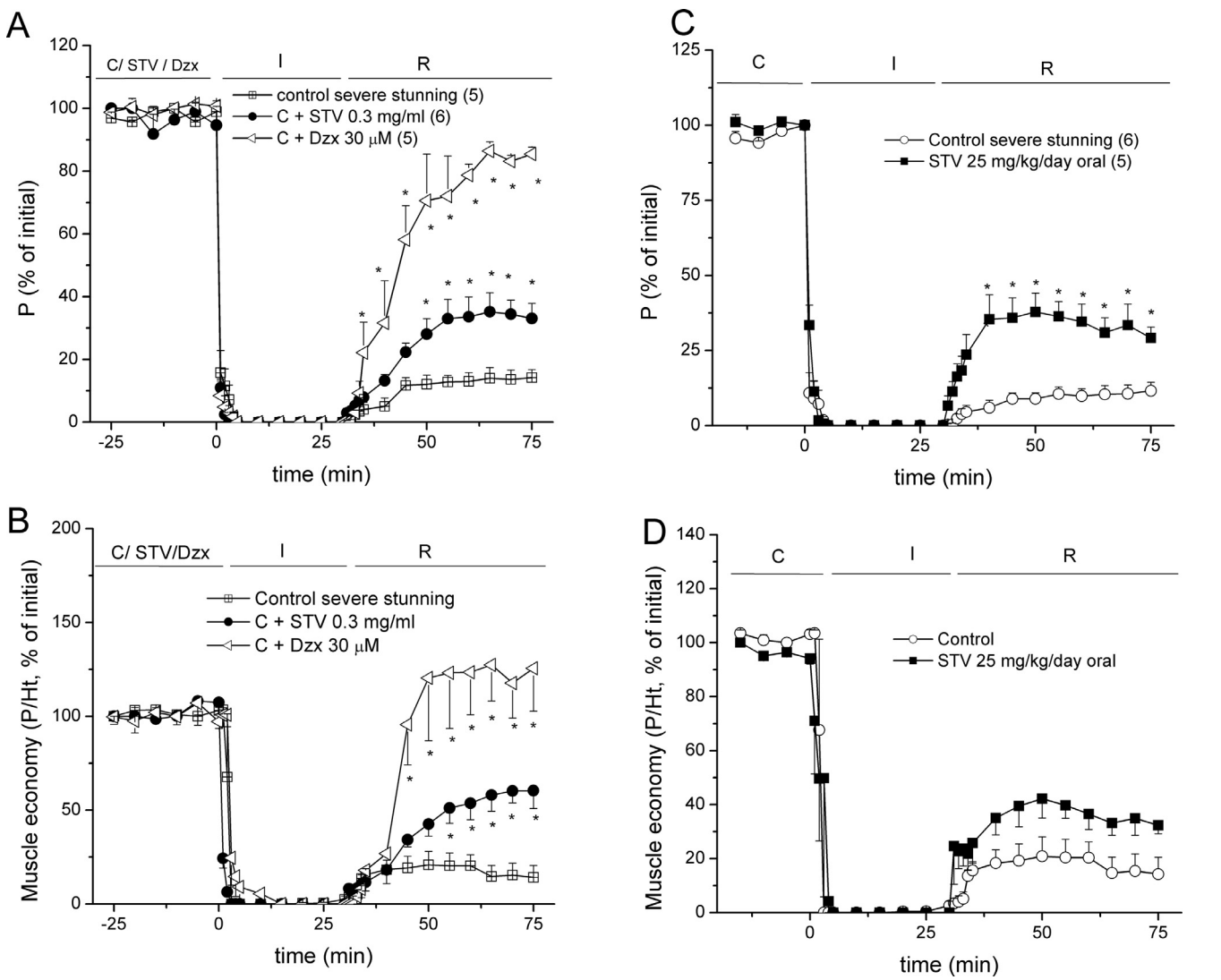

Fig. 2. Effects of stevioside (STV) on the severe stunning developed by 30 min ischemia (I) and 45 min reperfusion (R) at 37 °C in comparison with non-treated hearts (C, control). **A** and **B** show the effect of perfusing 0.3 mg/ml STV and the positive control 30 μM diazoxide (Dzx), while **C** and **D** show the effects of administering 25 mg/kg/day orally during one week before I/R. The upper graphs show maximal pressure development (P) (as % of the initial value), the lower graphs show muscle economy (P/Ht as % of initial). Results are shown as media ± SEM (*n* are indicated in panels A and C) (two-way ANOVA's for **A** and **B:** by treatment F = 154.2, and F = 80.37, both $p < 0.0001$, and by time: F = 296.6 and 67.61, both $p < 0.0001$, respectively; and for **C** and **D:** by treatment F = 144.4, $p < 0.0001$, and F = 8.959, p = 0.003, and by time: F = 196.8 and 26.93 both $p < 0.0001$, respectively; Post-tests $^{*}p < 0.05$ vs. Control).

RyR2 with the consequent massive release of Ca^{2+} from the SR proportionally to its content (Bers, 2001). The resultant contracture was accompanied by an increase in Ht, which can be attributed to a Ca^{2+} cycling between SERCA and RyR2. This is because Ca^{2+} can not be extruded by the sarcolemmal NCX at low $[Na^{+}]o$, but it can be removed by the mitochondrial uniporter, with the consequent dissipation of their electrochemical gradient, metabolism activation and heat release (Consolini et al., 2007). In comparison with the non-treated hearts (control) STV increased the AUC-ΔLVP associated to a slight reduction in the relaxation rate of the caffeine-induced contracture (Fig. 3A). This result suggests that Ca^{2+} was extruded more slowly from cytosol by the mitochondrial uptake, which is the main Ca^{2+} removal mechanism when SERCA and NCX are minimized. The fact that STV did not change Ht during the contracture suggests that the reduction in mitochondrial metabolism when Ca^{2+} is collected was compensated by a same increase in energetical consumption to remove the remaining cytosolic Ca^{2+}. In the cytosolic side, the heat released by SERCA futile cycling is equivalent to the enthalpy of ATP for moving 2 Ca^{2+} (40 mJ/μmol Ca^{2+}) while NCX is minimized but sarcolemmal Ca^{2+} ATPases could effectively remove Ca^{2+} by consuming 80 mJ/μmol Ca^{2+} (1 ATP for 1 Ca^{2+},). On the mitochondrial side, the energetic equivalent is 477 mJ/μmol O_2 or 80 mJ/μmol Ca^{2+} (since the electron transport chain moves 12 H^{+}/O_2, and 1 $Ca^{2+}/2H^{+}$) (Curtin and Woledge, 1978). Also, the actomyosin crosstalk during the contracture consumes ATP and releases heat (Mulieri and Alpert, 1982).

In the severe stunning model STV was more cardioprotective than in the moderate stunning one, with a definite increment in the PICR and muscle economy (compare Figs. 1 and 2). This effect is qualitatively similar to that obtained with the known cardioprotective diazoxide, which activates the KATP channels and consequently reduces the mitochondrial Ca^{2+} overload (Garlid et al., 2003). Nevertheless, perfusion of STV induced increase in LVEDP during I/R in both models but it was more important in the severe stunning. Considering that I/R increases the Ca^{2+} leak from SR (Valverde et al., 2010; Mattiazzi et al., 2015), the effect of STV on PICR and ΔLVEDP suggests that it may be increasing both, the SR Ca^{2+} content and the leak during I/R. In fact, the reperfusion with 10 mM caffeine-36 mM Na^{+}-Krebs increased the initial peak of contracture after treatment with STV, which suggests an increase in the SR Ca^{2+} store released by caffeine (Bers, 2001). In control conditions, the AUC-ΔLVP of hearts exposed to severe I/R was

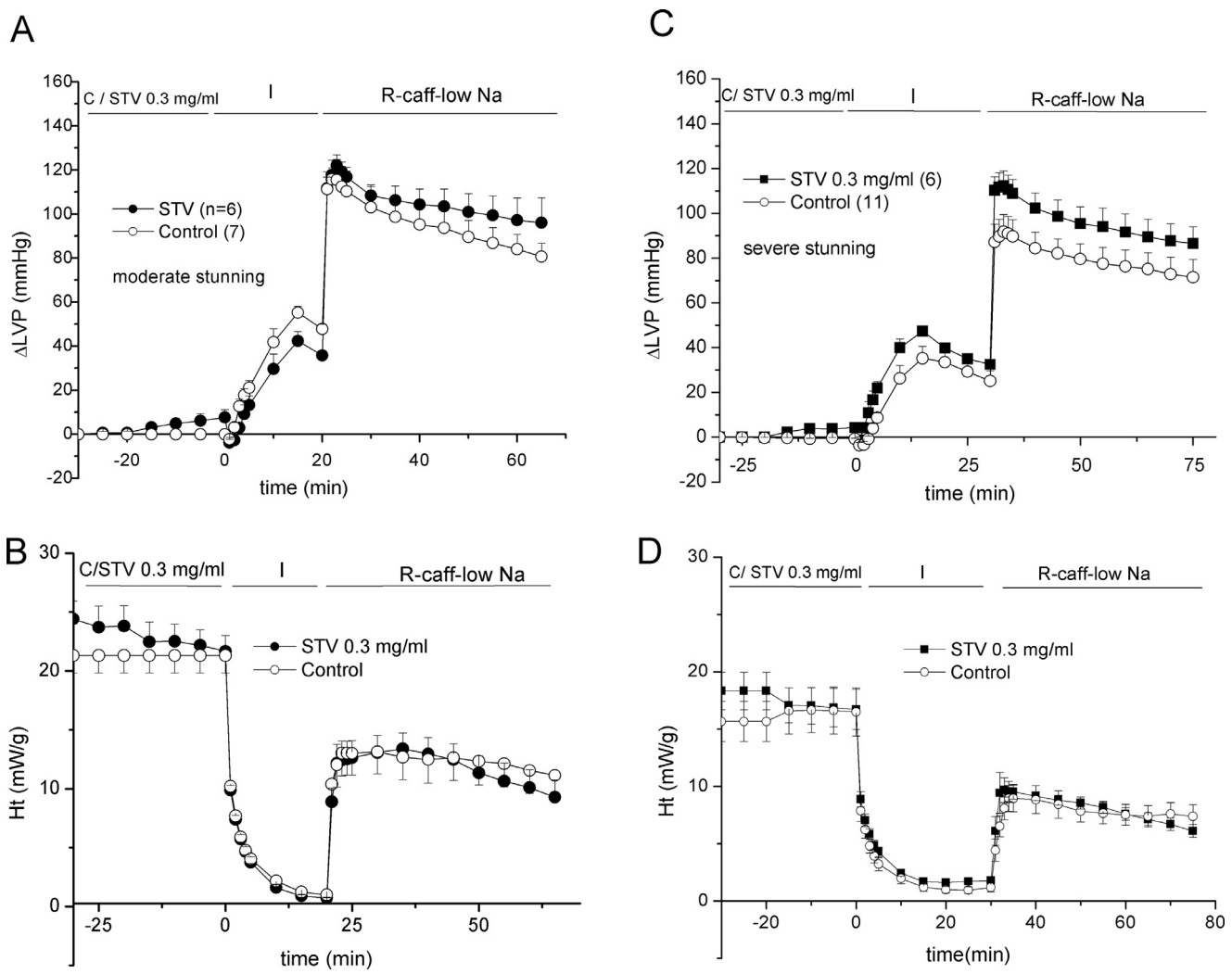

Fig. 3. Effects of 0.3 mg/ml stevioside (STV) on the sarcorreticular content estimated by postischemic reperfusion with 10 mM caffeine-36 mM Na-Krebs, in rat hearts exposed to 20 min ischemia (**A-B**) or to 30 min ischemia (**C-D**) at 37 °C. The changes on left ventricular pressure (ΔLVP) over the control preischemic condition (in mmHg) and (n) are shown in **A** and **C**, and the absolute values of heat rate (Ht in $mW.g^{-1}$) are shown in **B** and **D** (Two-way ANOVA's: for **A** and **B:** by treatment F = 1.868, $p = 0.17$, and F = 0.02, $p = 0.88$ NS; and by time: F = 144.9 and 45.65, both $p < 0.0001$, respectively; for **C** and **D** in severe stunning: by treatment F = 57.55, $p < 0.0001$, and F = 6.02, $p = 0.014$; and by time: F = 104.1 and F = 34.67, both $p < 0.0001$, respectively). Tests a posteriori: $^{*}p < 0.05$ vs. Control.

Table 2
Area under the curves of intraventricular pressure (ΔLVP) and heat rate (*ΔHt*) over the end of ischemia value, during the reperfusion with Krebs-C-10 mM caffeine- 36 mM Na^+ on rat hearts with the different treatments described in the text. (*$^{*}p < 0.05$* versus Control*)*.

Treatment	AUC-ΔLVP (mmHg min)	AUC-ΔHt (mW min/g)
Moderate stunning at 37 °C:		
Control (7)	2114.9 ± 349.6	504.5 ± 86.1
C + STV 0.3 mg/ml (6)	3063.6 ± 241.8*	493.4 ± 75.8
Severe stunning at 37 °C:		
Control (11)	2428.9 ± 193.7	297.6 ± 46.7
C + STV 0.3 mg/ml (6)	2838.0 ± 365.7	279.5 ± 20.9

similar to that obtained with moderate stunning, suggesting that the SR did not lose Ca^{2+} after 10 min more of ischemia (Table 2). STV increased the LVP during the whole period of I and R-caff low [Na^+] respect to the control group, and had a tendency to increase the AUC-ΔLVP. Simultaneously, Ht and AUC-Ht were similar for both groups of C and STV, again suggesting that the changes in Ca^{2+} movements were energetically equivalents.

When non-ischemic cardiomyocytes were perfused with 10 mM caffeine in a 36 mM Na^+-Krebs, cytosolic [Ca^{2+}] (F/Fo Fluo-4) was increased and then reduced at the time in which the mitochondrial [Ca^{2+}] (F/Fo Rhod-2) was raised (Fig. 4). These responses show that after the caffeine-dependent SR release the cytosolic Ca^{2+} was removed towards mitochondria. Perfusion of STV during the caffeine treatment did not change the pattern of cytosolic [Ca^{2+}]. Nevertheless, mitochondrial [Ca^{2+}] was early increased by STV but afterwards it was reduced regarding control cells. This pattern suggests that in non-ischemic myocytes STV increased both, mitochondrial Ca^{2+} uptake by UCam and Ca^{2+} extrusion by mNCX. We have previously described that this pathway provides the functional interaction between mitochondria and the SR store to regulate PICR (Ragone and Consolini, 2009). The mitochondrial Ca^{2+} uptake activates metabolism, and consequently the oxidative phosphorylation, explaining that reperfused hearts previously treated with STV have a higher muscle economy (P/Ht) in all models. Nevertheless, the contracture obtained by reperfusing ischemic hearts with 10 mM caffeine-low [Na^+] Krebs was more slowly relaxed after STV treatment, suggesting that ischemia changed the STV effect to attenuate the mitochondrial Ca^{2+} uptake. Since mitochondria and SR determine a balance of Ca^{2+} movements, the reduced mitochondrial uptake becomes in increased SR Ca^{2+} store, thus raising the Ca^{2+}-induced Ca^{2+} release and the force of contraction. The cardioprotection of STV was greater in the model of severe stunning in rats orally treated

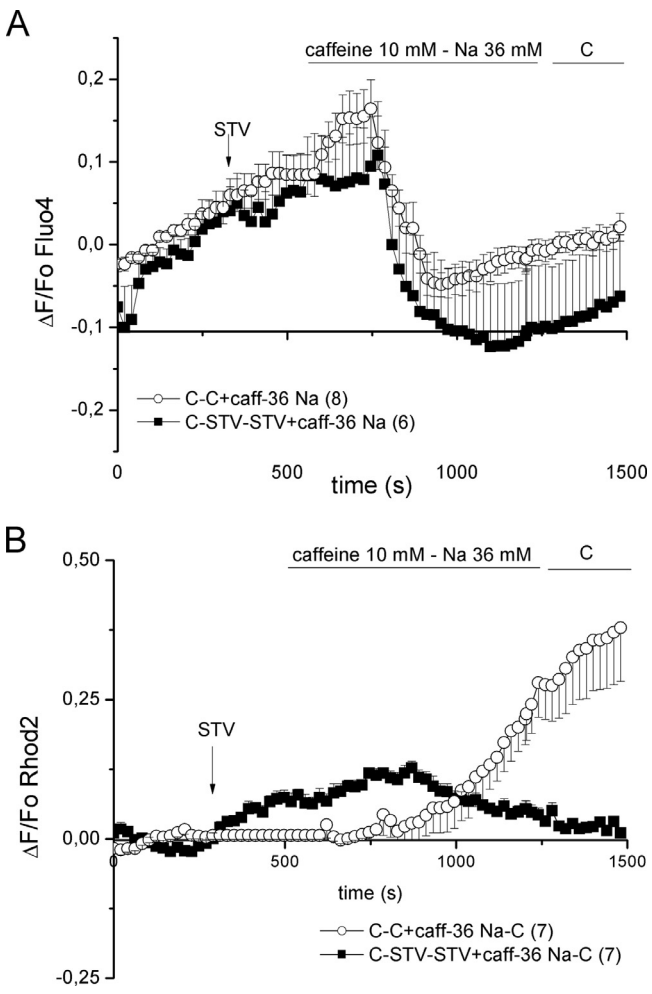

Fig. 4. Effects of 0.3 mg/ml stevioside (STV) on the changes in free [Ca^{2+}] induced in non-ischemic rat cardiomyocytes by perfusing 10 mM caffeine-36 mM Na-Krebs measured by confocal microscopy. The relative changes in fluorescence (ΔF/Fo) of Fluo-4 to estimate cytosolic [Ca^{2+}] (**A**) and Rhod-2 to estimate mitochondrial [Ca^{2+}] (**B**). Compare the conditions with (STV) and without (C) stevioside.

with 25 mg/kg/day STV, since it increased PICR without important diastolic contracture (Fig. 2 and Table 1). These effects suggest that oral STV increased the SR Ca^{2+} store with a reduced leak. The differences between both models of STV administration previous to severe ischemia, suggest that part of the effect of STV may be due to a metabolite, such as the described isosteviol, which would reduce the SR Ca^{2+} leak induced by STV. It was reported a human doses of 1 g stevioside/day in diabetic patients, equivalent to 17 mg/kg for a patient of 60 kg, which resulted in a plasmatic concentration of about 0.33 mg STV/ml (Gregersen et al., 2004). Thus, the oral doses of 25 mg/kg/day is equivalent to that used in humans, as well as the concentration used for perfusing the isolated hearts was equivalent to the plasmatic concentration. In conclusion, this work shows that STV is a cardioprotective agent, which increases the sarcorreticular Ca^{2+} release, and could be preventive of stunning for patients that have any risk factor of coronary dysfunction which causes a transient period of ischemia and reperfusion.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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